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ORIGINAL ARTICLE

Multiplex real-time PCR (TaqMan) assay for the simultaneous detection and discrimination of potato powdery and common scab diseases and pathogens

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detection, potato, real-time PCR, *Spongospora subterranea*, *Streptomyces*.

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Abstract

Aims: To develop a multiplex real-time PCR assay using TaqMan probes for the simultaneous detection and discrimination of potato powdery scab and common scab, two potato tuber diseases with similar symptoms, and the causal pathogens *Spongospora subterranea* and plant pathogenic *Streptomyces* spp.
Methods and Results: Real-time PCR primers and a probe for *S. subterranea* were designed based on the DNA sequence of the ribosomal RNA ITS2 region. Primers and a probe for pathogenic *Streptomyces* were designed based on the DNA sequence of the *txtAB* genes. The two sets of primer pairs and probes were used in a single real-time PCR assay. The multiplex real-time PCR assay was confirmed to be specific for *S. subterranea* and pathogenic *Streptomyces*. The assay detected DNA quantities of 100 fg for each of the two pathogens and linear responses and high correlation coefficients between the amount of DNA and C_t values for each pathogen were achieved. The presence of two sets of primer pairs and probes and of plant extracts did not alter the sensitivity and efficiency of multiplex PCR amplification. Using the PCR assay, we could discriminate between powdery scab and common scab tubers with similar symptoms. Common scab and powdery scab were detected in some tubers with no visible symptoms. Mixed infections of common scab and powdery scab on single tubers were also revealed.
Conclusions: This multiplex real-time PCR assay is a rapid, cost efficient, specific and sensitive tool for the simultaneous detection and discrimination of the two pathogens on infected potato tubers when visual symptoms are inconclusive or not present.
Significance and Impact of the Study: Accurate and quick identification and discrimination of the cause of scab diseases on potatoes will provide critical information to potato growers and researchers for disease management. This is important because management strategies for common and powdery scab diseases are very different.

Introduction

Common scab and powdery scab are common blemish diseases of potato tubers worldwide. Scab lesions on the tuber skin seriously reduce tuber quality and marketability, resulting in significant economic loss. Common scab is caused by pathogenic bacteria belonging to several species in the genus *Streptomyces* (Loria *et al.* 2006; Wanner 2009). *Streptomyces* is the most widely studied and well-known genus of *Actinobacteria*, Gram-positive

filamentous bacteria that are predominantly soil saprophytes. Of the more than 400 *Streptomyces* spp. described, only a few are known to be pathogenic to the underground portions of a range of host plants including potato, carrot, radish, beet and other tap root crops (Goyer and Beaulieu 1997; Loria *et al.* 1997). Among the plant diseases caused by *Streptomyces*, potato common scab is the most economically important (Lambert and Loria 1989; Loria *et al.* 1997; Hill and Lazarovits 2005). Powdery scab is caused by the protist *Spongospora subterranea. Spongospora subterranea* is a biotrophic pathogen classified as a member of the Plasmodiophorales, which are characterized as having multinucleate plasmodia and biflagellate zoospores and resting spores (Karling 1968; Harrison *et al.* 1997). These two pathogens are both seed- and soilborne. They can be spread on infected seed tubers with subsequent build-up of inoculum levels in soils. Current control measures for common scab and powdery scab are limited. There is no effective chemical treatment, and most popular potato cultivars have little resistance (Powelson *et al.* 1993). The ideal control would be to plant certified, scab-free seed of resistant potato cultivars in clean soils.

While common scab and powdery scab are caused by very different pathogens and the conditions favouring each disease are different, the symptoms of the two diseases on potato tubers are similar and can be very difficult to distinguish with the naked eye. Typical common scab symptoms include brown irregularly shaped, raised or depressed cork-like blemishes on the tuber skin. Powdery scab symptoms on tubers are typically raised pustules with a central depression harbouring a powdery spore mass. However, depending on potato cultivar, environmental conditions and growing locations, symptoms of common scab and powdery scab can be quite variable and indistinguishable, even for the potato disease specialist. Accurate identification of the cause of diseases on potatoes is required in screening potato cultivars and breeding lines for resistance and for providing information to potato growers and researchers who request assistance in diagnoses. A rapid, cost efficient, specific, sensitive method is therefore needed for the simultaneous detection of the two pathogens, enabling disease identification and discrimination.

PCR assays have been developed for the specific detection of pathogenic *Streptomyces* spp. (Cullen and Lees 2007; Qu *et al.* 2008) and *S. subterranea* (Bulman and Marshall 1998; van de Graaf *et al.* 2003; Qu *et al.* 2006), respectively. However, none of the published protocols allows for the simultaneous detection of these two pathogens on potato tubers. A reliable and efficient multiplex detection system that differentiates powdery and common scab would save researchers', clinicians' and certified seed producers' time and money. This paper describes the development of a multiplex real-time PCR assay using TaqMan probes for the simultaneous detection of *S. subterranea* and pathogenic *Streptomyces* spp.

Materials and methods

Spongospora subterranea isolates and *Streptomyces* strains

Isolates of *S. subterranea* and *Streptomyces* strains analysed in this study are listed in Table 1. Twenty-four field isolates of *S. subterranea* were obtained either from different fields or from the same field with samples taken over several years in the United States and Canada. Each isolate was prepared by the removal of single spore balls from powdery scab lesions of naturally infected potato tubers by the method of Qu *et al.* (2001). Sixteen *Streptomyces* strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) or were isolated from scab lesions on potato tubers according to the method of Wanner (2004) and tested for pathogenicity on radishes and/or potatoes as described previously (Wanner 2004, 2007). *Streptomyces* species determinations were made by cloning and sequencing the 16S ribosomal RNA gene and comparing the *rrn* sequences obtained to sequences deposited in GenBank (Wanner 2006, 2007, 2009; L.A. Wanner, unpublished data).

Plant material and soil samples

Seventy-nine tuber samples from potato fields where powdery scab and/or common scab are found were used in this study. The tuber samples were either collected from our disease resistance screening programmes in Pennsylvania or sent to us by potato growers or researchers from other states in the United States for the diagnosis of scab diseases. Scab symptoms on tubers were examined visually. Some samples with typical powdery scab or common scab symptoms were used as positive controls but most samples had atypical scab symptoms and were subjected to real-time PCR analyses in this study. Pathogen-free mini-tubers cv. Russet Burbank and Atlantic (Summit Plant Laboratories, Inc., Fort Collins, CO, USA) were used as negative controls. Tubers were washed gently under running tap water to remove soil particles from the surface. For symptomatic tubers, 1 g of scab lesion tissue was used for DNA extraction. For asymptomatic tubers, 1 g of peel tissue was used for DNA extraction. Twelve soil samples were collected from a potato field at Potter County, PA, USA at harvest in 2008. Powdery scab and common scab have previously been observed on potatoes in this field.

Table 1 Description of *Spongospora subterranea* and *Streptomyces* spp. isolates used in this study and specificity of multiplex real-time PCR assay

Genus and species*	Origin	Pathogenicity†	Multiplex real-time PCR‡	
			CAL Fluor Red610§	FAM¶
Spongospora subterranea	Potato tuber, typical powdery scab lesion; ME	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ME	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ME	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CO	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CO	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CO	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CO	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; PA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ID	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ID	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ID	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; ID	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; NY	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; NY	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; CA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion, WA	N/A	–	+
S. subterranea	Potato tuber, typical powdery scab lesion; New Brunswick, Canada	N/A	–	+
Streptomyces scabies	Potato tuber, typical common scab lesion; ME	+	+	–
Streptomyces glauciniger	Potato tuber, atypical lesion; ME	–	–	–
Ochrobactrum anthropii	Potato tuber, netted surface; ME	–	–	–
Streptomyces coelescens	Potato tuber, typical common scab lesion; ME	–	–	–
Streptomyces clavifer; *Streptomyces griseus*	Potato tuber, typical common scab lesion; ND	–	–	–
Streptomyces griseus; *Streptomyces setonii*	Potato tuber, typical common scab lesion; WI	–	–	–
Streptomyces badius; *Streptomyces sindensis*	Potato tuber, typical common scab lesion; ID	–	–	–
Streptomyces stelliscabiei	Potato tuber, typical common scab lesion; NY	+	+	–
Streptomyces spp. IdX	Potato tuber, typical common scab lesion; ID	+	+	–
S. scabies	Potato tuber, typical common scab lesion; OH	+	+	–
S. scabies (type strain, ATCC 49173)	Potato tuber; NY	+	+	–
S. europaeiscabiei	Potato tuber, typical common scab lesion; ID	+	+	–
S. scabieis (ATCC 33282)	Soil; unknown location	+	+	–
Streptomyces turgidiscabies (type strain, ATCC 700248)	Potato tuber, Hokkaido, Japan	+	+	–
Streptomyces aureofaciens (type strain, ATCC 700526)	Potato tuber scab lesion, Loimaa, Finland	–	–	–
Streptomyces acidiscabies (type strain, ATCC 49003)	Potato tuber, ME	+	+	–

+, Positive; –, negative.

*Or most similar species, *Streptomyces* spp. based on sequence of the 16s rRNA gene.

†*Streptomyces* spp. pathogenicity tested on radishes and/or potatoes (Wanner 2004, 2007).

‡The threshold setting was default 30 fluorescent units and used for determining the crossing points C_t values.

§Pathogenic *Streptomyces* spp.-specific primers and probe targeted on operon *txtAB*.

¶*Spongospora subterranea*-specific primers and probe targeted on rDNA ITS2 region.

DNA extraction

DNA from *S. subterranea* spore balls was extracted by the method of Möller and Harling (1996). DNA from potato tuber tissues (1 g per sample) was extracted using the hexadecylmethylamonium bromide method (Doyle and Doyle 1990) and redissolved in 200 μl sterilized distilled water. *Streptomyces* strains were grown at 28°C for 3 days in yeast-malt extract (Schaad *et al.* 2001), and cells were pelleted to obtain approximately 100-mg aliquots that were stored at −20°C until use. DNA was isolated from frozen cells using a Bio101 FAST DNA kit (MP Biomedicals, Solon, OH, USA). DNA was extracted from soil using an UltraClean™ Soil DNA kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA). DNA concentrations were determined using a GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK).

Primer and TaqMan probe design

We previously designed real-time PCR primers StrepF and StrepR based on *txtAB* gene sequences of *Streptomyces acidiscabies* AF255732 and *Streptomyces turgidiscabies* AY707081. The *txtAB* gene encodes thaxtomin synthetase and has been found in a large chromosomal region that functions as a pathogenicity island in *Streptomyces* (Kers *et al.* 2005). The real-time PCR assay using SYBR Green and these primers was confirmed to be specific for pathogenic *Streptomyces* (Qu *et al.* 2008). In this study, a TaqMan probe StrepP (Table 2) was designed based on *txtAB* gene sequences of *S. acidiscabies* and *S. turgidiscabies* between the StreptF and StreptR primers using Primer Express® software ver. 2.0 (Applied Biosystems, Foster City, CA, USA). The probe sequence was compared with the NCBI nonredundant nucleotide sequence database using Blastn and showed no identity with sequences from other organisms. The StrepP probe was manufactured with a CAL Fluor Red610 (Bioresearch Technologies, Novato, CA) at the 5′ end as the reporter dye and BHQ-2 at the 3′ end as the quencher.

Spongospora subterranea-specific real-time PCR primers SponF and SponR and TaqMan probe SponP (Table 2) were designed based on the rDNA sequence of the ITS2 region of *S. subterranea* using Primer Express® software ver. 2.0 (Applied Biosystems). There are two types of intergenic transcribed spacer (ITS) sequences of *S. subterranea* (AY604171 and AY604172), and the primers and probes were designed from a region of 100% identity between the two. The sequences of primers SponF, SponR and probe SponP were compared with the NCBI nonredundant nucleotide sequence database using Blastn and showed no identity with sequences from other organisms including closely related plasmodiophorid *Plasmodiophora brassicae*, *Polymyxa* species and *Spongospora nasturtii*. The probe SponP was 5′ labelled with FAM as the reporter dye, and the 3′ end was labelled with BHQ-1 as the quencher. A third real-time PCR primer pair CoxF/R and TaqMan probe CoxP (Table 2) were designed to amplify the potato cytochrome oxidase gene, as described by Weller *et al.* (2000). These were used as a control to detect potato DNA. The probe CoxP was 5′ labelled with FAM as the reporter dye and 3′ labelled with BHQ-1 as the quencher. All probes were manufactured by Biosearch Technologies (Novato, CA, USA).

Real-time PCR amplification

The multiplex real-time PCR assay using primer pairs StrepF/R and SponF/R and probes StrepP and SponP was optimized initially using pure *Streptomyces scabies* and *S. subterranea* DNA templates. The parameters tested included the concentrations of $MgCl_2$ (2·5–6·5 mmol l^{-1}), primers (0·1–1 μmol l^{-1}), probes (0·1–1 μmol l^{-1}) and DNA templates. Based on the results of these tests, an optimized multiplex real-time PCR amplification was performed in a 25-μl reaction mixture containing 1× PCR

Table 2 Real-time PCR primers and probes used in this study

Target organism	Primer and probe	Sequences (5′ → 3′)	T_m* (°C)	Target gene	Product size (bp)	References
Spongospora subterranea	SponF	CTTTGAGTGTCGGTTTCTATTCTCCC	61·4	ITS2 region	138	This work
	SponR	GCACGCCAATGGTTAGAGACG	61·5			
	SponP	FAM-TCTTTCAAGCCATGGACCGACCAGA-BHQ-1	68·4			
Pathogenic *Streptomyces* spp.	StrepF	GCAGGACGCTCACCAGGTAGT	60·3	*txtAB*	71	Qu *et al.* (2008); This work
	StrepR	ACTTCGACACCGTTGTCCTCAA	60·5			
	StrepP	CAL Fluor Red610-TCGGTGATCCA GTACTTTCCGTCGGC-BHQ-2	70·3			
Potato	CoxF	CGTCGCATTCCAGATTATCCA	59·5	*cox*	79	Weller *et al.* (2000)
	CoxR	CAACTACGGATATATAAGAGCCAAAACTG	59·7			
	CoxP	FAM-TGCTTACGCTGGATGGAATGCCCT-BHQ-1	68·2			

*Melting temperature (T_m) determined by Primer Express version 2.0 software (Applied Biosystems).

buffer (Applied Biosystems), 6 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP, 0·1 μmol l^{-1} of each of four primers, 0·1 μmol l^{-1} of probe SponP, 0·3 μmol l^{-1} of probe StrepP and 0·625 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Ten nanograms of *Streptomyces* or *S. subterranea* DNA or 10 μl of 1/50 diluted tuber DNA extract or 10 μl of 1/40 diluted soil DNA extract was used as DNA template per reaction. Real-time PCR amplification was carried out on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) with an initial activation step at 95°C for 10 min, followed by 45 cycles of 95°C for 25 s and 60°C for 1 min. Fluorescence was measured during the annealing step of each cycle. The threshold setting was default 30 fluorescent units and used for determining the crossing points (C_t values). PCR efficiency was determined from the slope of a calibration dilution curve by the equation $E = 10^{[-1/\text{slope}]} - 1$, with $E = 1{\cdot}000$ being perfect. In the initial tests, the PCR products were separated by electrophoresis on 1·5% agarose gels to verify the size of the amplified product and to check for the presence of primer dimers or nonspecific bands. Single-plex real-time PCR assays were performed as described above with only one set of primers and probe in the amplification reaction.

The specificity of the multiplex real-time PCR assay was evaluated using DNA from mini-tuber tissues and from common potato tuber pathogens *Alternaria solani*, *Colletotrichum coccodes*, *Fusarium sambucinum*, *Helminthosporium solani* and *Phytophthora infestans*.

Results

Specificity of the multiplex real-time PCR assay

The use of the multiplex real-time PCR assay with two sets of primer pairs and probes to detect and distinguish between *S. subterranea* and pathogenic *Streptomyces* was evaluated (Table 1). DNA from 24 *S. subterranea* isolates and nine known pathogenic and eight known nonpathogenic *Streptomyces* strains was used. FAM fluorescence was detected only in DNA from each of the 24 *S. subterranea* isolates. CAL Fluor Red610 fluorescence was detected only in DNA from each of nine pathogenic *Streptomyces* strains. No cross-reactivity was observed. No FAM or CAL Fluor Red610 fluorescence was detected in any of DNA samples from pathogen-free mini-tuber tissues or from common potato tuber pathogens *A. solani*, *C. coccodes*, *F. sambucinum*, *H. solani* and *P. infestans*.

Sensitivity of the multiplex real-time PCR assay

The sensitivity and detection range of the multiplex real-time PCR assay using two sets of primer pairs and probes were determined using a tenfold dilution series of DNA from pathogenic *Streptomyces* and *S. subterranea* ranging from 10 ng to 10 fg. C_t values were variable for three replications for 10 fg of either genomic *Streptomyces* DNA or *S. subterranea* DNA and, therefore, not included in the analysis. C_t values were similar for three replications for 100 fg of each pathogen DNA, and therefore, the reliable lower limit of detection was 100 fg of genomic *Streptomyces* DNA and *S. subterranea* DNA. The C_t values of the multiplex real-time PCR assay are shown in Table 3. Based on multiplex PCR assay of three replicate serial dilutions, a standard curve was constructed by plotting C_t values *vs* the log of the initial concentration of DNA for each pathogen. For *S. subterranea* DNA, the standard curve showed a high correlation coefficient ($R^2 = 0{\cdot}996$) with a slope of −3·385 (Fig. 1a) and PCR efficiency was 0·974, indicating a reproducible linear response in detection of the pathogen DNA. For pathogenic *Streptomyces* DNA, the standard curve also showed a high correlation

Table 3 Real-time multiplex PCR C_t values for pathogen (*Spongospora subterranea* and pathogenic *Streptomyces* spp.) DNA alone and for pathogen DNA plus potato DNA

Pathogen	Amount of pathogen DNA	C_t values: Pathogen DNA alone	C_t values: Pathogen DNA plus 0·98 ng potato DNA
S. subterranea	10 ng	19·38 ± 0·29	19·54
	1 ng	22·37 ± 0·24	22·46
	100 pg	25·74 ± 0·19	26·12
	10 pg	29·34 ± 0·60	28·89
	1 pg	32·62 ± 0·57	33·04
	100 fg	36·09 ± 0·43	36·74
Pathogenic *Streptomyces* spp.	10 ng	23·95 ± 0·25	24·14
	1 ng	27·58 ± 0·21	28·10
	100 pg	30·87 ± 0·29	30·84
	10 pg	33·83 ± 0·21	34·16
	1 pg	37·31 ± 1·00	36·80
	100 fg	39·47 ± 1·61	39·98

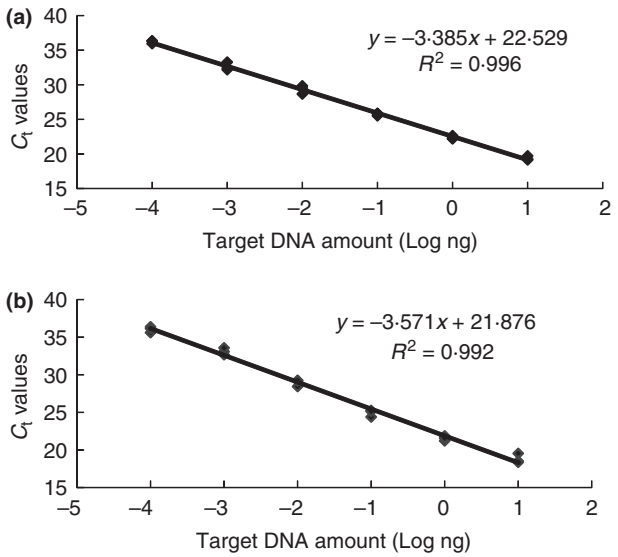

Figure 1 Standard curve obtained by plotting threshold cycle (C_t) values against log serial dilutions of DNA (ng) from *Spongospora subterranea*. The standard curve is the result of three separate PCR analyses. (a) Multiplex real-time PCR. (b) Single-plex real-time PCR.

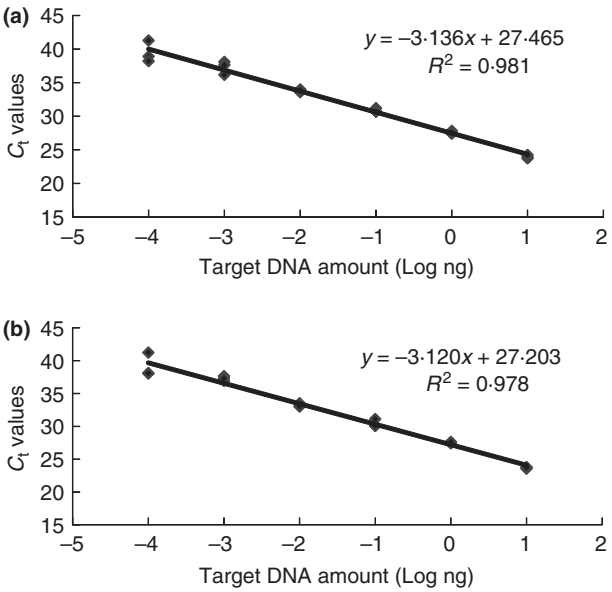

Figure 2 Standard curve obtained by plotting threshold cycle (C_t) values against log serial dilutions of DNA (ng) from pathogenic *Streptomyces* spp. The standard curve is the result of three separate PCR analyses. (a) Multiplex real-time PCR. (b) Single-plex real-time PCR.

coefficient (R^2 = 0·981) with a slope of −3·136 (Fig. 2a) and PCR efficiency was 1·084.

Because the presence of the other oligonucleotides and fluorescent dyes could alter the efficiency of multiplex real-time PCR amplification, each set of primers and probe was tested in parallel in single-plex real-time PCR using dilution series of pathogenic *Streptomyces* and *S. subterranea*. For *S. subterranea* DNA, the standard curve showed a high correlation coefficient (R^2 = 0·992) with a slope of −3·571 (Fig. 1b) and PCR efficiency was 0·906. For pathogenic *Streptomyces* DNA, the standard curve showed a high correlation coefficient (R^2 = 0·978) with a slope of −3·120 (Fig. 2b) and PCR efficiency was 1·092. Thus, there was no systematic deviation in the amplification curves when comparing the multiplex PCR assay with the single-plex PCR assays for each pathogen, and no significant difference in amplification efficiency was found as measured by the slopes of standard curves. In addition, the respective detection limits and ranges for the single-plex and multiplex PCR assays were identical, and the most dilute samples (100 fg) were detected in both types of assays.

When 0·98 ng of DNA from mini-tuber cv. Russet Burbank was added to serial dilutions of pathogenic *Streptomyces* or *S. subterranea* DNA, C_t values for each dilution were similar to those obtained from serial dilutions of clean pathogen DNA without added plant DNA extracts (Table 3). For *S. subterranea* DNA plus potato DNA, the standard curve showed a high correlation coefficient (R^2 = 0·997) with a slope of −3·443 and PCR efficiency was 0·951. For pathogenic *Streptomyces* DNA plus potato DNA, the standard curve also showed a high correlation coefficient (R^2 = 0·997) with a slope of −3·103 and PCR efficiency was 1·100. Because the presence of plant DNA and co-extracts did not affect the amplification of target DNA, this multiplex real-time PCR can be used to detect pathogenic *Streptomyces* and *S. subterranea* in potato tubers.

Analysis of field tuber samples

Of 79 field potato tuber samples examined visually, seven showed typical symptoms of common scab, six showed typical symptoms of powdery scab, 60 showed symptoms that were difficult to diagnose as either powdery or common scab (or other scab-like symptoms) and six showed no scab symptoms (Table 4). DNA from tuber samples was amplified by the multiplex real-time PCR assay, and the results are shown in Table 4. Pathogenic *Streptomyces* was detected in DNA extracts from all seven tubers with typical common scab symptoms and C_t values ranged between 21·54 and 25·49, and no *S. subterranea* was detected in these extracts. *Spongospora subterranea* was detected in DNA from all six typical powdery scab tubers and C_t values ranged between 19·82 and 29·47, and no pathogenic *Streptomyces* was detected in these extracts. Of the 60 tuber samples with unclear scab symptoms, 30

Table 4 The results of multiplex real-time PCR evaluation of 79 field potato tuber samples and 12 potato field soil samples for the presence of *Spongospora subterranea* and pathogenic *Streptomyces* spp.

Samples	Total	Pathogenic *Streptomyces* positive only	*S. subterranea* positive only	*S. subterranea* and pathogenic *Streptomyces* positive	*S. subterranea* and pathogenic *Streptomyces* negative
Typical common scab tuber	7	7	0	0	0
Typical powdery scab tuber	6	0	6	0	0
Atypical scab tuber	60	30	14	11	5
Asymptomatic tuber	6	0	1	3	2
Mini-tuber*	2	0	0	0	1
Soil	12	6	0	5	1

*Pathogen-free mini-tubers cv. Russet Burbank and Atlantic used as negative controls.

showed the presence of pathogenic *Streptomyces* only with C_t values ranged between 22·89 and 35·72, 14 showed the presence of *S. subterranea* only with C_t values ranged between 24·11 and 37·34, 11 showed both pathogens with *S. subterranea* C_t values ranged between 29·27 and 37·45 and pathogenic *Streptomyces* C_t values ranged between 26·46 and 38·58, and five showed neither pathogen. Of the six tuber samples with no scab symptoms, *S. subterranea* only was detected in one DNA extract with a C_t value of 33·64, both pathogens were detected in three samples with *S. subterranea* C_t values ranged between 34·06 and 38·51 and pathogenic *Streptomyces* C_t values ranged between 29·38 and 40·17. Neither pathogen was detected in the other two samples. No pathogenic *Streptomyces* or *S. subterranea* was detected on negative control mini-tuber samples. For samples negative for both pathogens, a second single-plex real-time PCR assay using potato primer pair CoxF/R and probe CoxP was performed. Fluorescence signal was detected in all of these samples (data not shown), indicating that all extracted potato DNA was amplifiable and of good quality and that these samples were either free of pathogenic *Streptomyces* and *S. subterranea* or the amount of pathogen DNA in the samples was below the reliable detection limit (100 fg).

Analysis of field soil samples

Multiplex real-time PCR assay was carried out on DNA from 12 potato field soil samples to determine whether the assay could be used for pathogen detection on soil samples. The results are shown in Table 4. *Spongospora subterranea* was detected in five samples with C_t values ranged between 34·15 and 36·85. Pathogenic *Streptomyces* was detected in 11 samples with C_t values ranged between 33·3 and 37·21.

Discussion

In this investigation, a multiplex real-time PCR assay using TaqMan probes was developed for the simultaneous detection of *S. subterranea* and pathogenic *Streptomyces* spp. The assay was specific and sensitive and was used to identify the presence of *S. subterranea* and/or pathogenic *Streptomyces* on field potato tubers. This PCR multiplex assay will provide a reliable, rapid and efficient tool for economic differential diagnoses of powdery scab and common scab pathogens on potatoes. We are using the assay to detect both pathogens in soil samples.

The *S. subterranea*-specific primers and probe were designed from the sequence of the ribosomal RNA ITS. The ITS region was chosen as the target sequence because there is low DNA sequence variation reported in *S. subterranea* isolates collected around the world. Two types of *S. subterranea* ITS sequences have been reported in North America, Europe and Asia. As only 2·9% sequence divergence was found between the two types (Bulman and Marshall 1998; Qu and Christ 2004), it was possible to design primers and probe based on an identical region of ITS2 sequence. Additionally, because rRNA genes are present in multiple copies per genome, use of this sequence increases the sensitivity of detection. Real-time PCR products were amplified in DNA extracts from 24 *S. subterranea* isolates from different locations in the United States, but not from DNA extracts from potato or several fungal or bacterial pathogens of potato, indicating the *S. subterranea* primers and probe are specific.

Primers and probe specific to pathogenic *Streptomyces* were designed based on *txtAB* gene sequences. *TxtAB* PCR products were amplified from nine pathogenic *Streptomyces* strains previously determined to be pathogenic that represent most species of plant pathogenic *Streptomyces* so far reported to occur in Europe and North America (Bukhalid and Loria 1997; Kreuze *et al.* 1999; Bouchek-Mechiche *et al.* 2006; Wanner 2006, 2007, 2009) but not from nonpathogenic *Streptomyces* strains and other organisms. *TxtAB* encodes thaxtomin synthetase, and the phytotoxin thaxtomin produced by pathogenic *Streptomyces* strains is the only known pathogenicity determinant for common scab diseases of potato and other root and tuber crops (Loria *et al.* 2006). A 100%

correlation between pathogenicity and the production of thaxtomin on oatmeal agar or the presence of thaxtomin biosynthetic *txtAB* genes has been reported (Bukhalid *et al.* 1998; Wanner 2004, 2006, 2009; Wang and Lazarovits 2005; Loria *et al.* 2006). Although primers developed on multicopy genes such as the 16S rRNA may improve sensitivity, it is not possible to design primers specific for all pathogenic strains while excluding the much more numerous nonpathogenic strains because both pathogenic and non-pathogenic strains are found within a single species.

Using serial dilutions of target DNA, the multiplex PCR assay detected DNA quantities of 100 fg for both pathogens, and linear responses and high correlation coefficients between the amount of DNA and C_t values for each pathogens were achieved. The C_t values, reliable detection limits and correlation coefficients of the multiplex real-time PCR were similar to that of single-plex real-time PCR. This result shows that the presence of two sets of primer pairs and probes did not alter the sensitivity and efficiency of the multiplex PCR amplification. Furthermore, the presence of plant extracts in the amplification mix in the amount equal to what would be used in assays of field tuber samples did not alter the sensitivity of the assay. These results indicate that the multiplex real-time PCR described here is appropriate for simultaneous detection of the two pathogens in potato tubers.

Control measures for plant diseases depend on proper identification of diseases and of the causal agents. Powdery scab and common scab are two important tuber blemish diseases of potatoes worldwide. Symptoms of the two scab diseases may be quite similar and difficult to distinguish even by potato experts, but pathogens and control measures for the two diseases are totally different. Of 60 field tuber samples with unclear scab symptoms, 30 scab tubers (50%) were shown by multiplex real-time PCR to be infected with pathogenic *Streptomyces* only and could therefore be identified as common scab tubers. Fourteen tubers (23·3%) were infected with *S. subterranea* only and could therefore be identified as powdery scab tubers. Accurate identification and discrimination of the pathogens on harvested tubers will help growers initiate long-term control procedures to avoid making the problem worse.

Common scab and powdery scab typically develop under different environmental conditions. Warm temperatures (20–30°C) and dry soil conditions favour common scab development on tubers, while cool temperatures (12–18°C) and wet soil conditions favour the development of powdery scab (van de Graaf *et al.* 2005). Mixed common scab and powdery scab infections of potato tubers were previously thought to be rare. In this study of 60 field tuber samples with scab symptoms, 11 (18·3%) were confirmed to be infected with both *S. subterranea* and pathogenic *Streptomyces* by the real-time PCR assay, indicating that mixed infection is not rare in the United States. More research is needed to understand the interaction between the two pathogens in plants and how mixed infection occurs.

Five of 60 scabby tubers (8·3%) were *S. subterranea*- and pathogenic *Streptomyces*-free, as evaluated by the real-time PCR assay. This result indicates that the scab symptoms on these tubers were not caused by *S. subterranea* or pathogenic *Streptomyces*. These symptoms may be physical disorders or may be caused by other pathogens such as *Rhizoctonia solani*. We are in the process of developing multiplex real-time PCR assays to detect additional potato tuber pathogens including *R. solani* (cause of sprout death, stem cankers and black scurf on tubers), *C. coccodes* (cause of black dot on tubers) and *H. solani* (cause of silver scurf on tubers).

Pathogenic *Streptomyces* and/or *S. subterranea* were detected on four of six asymptomatic field tuber samples, as determined by the multiplex real-time PCR assay. Although the sample size of six is quite small, our results show that both pathogens can be present on symptomless tubers. These two pathogens can both be seed-borne (Harrison *et al.* 1997; Wilson *et al.* 1999; Wang and Lazarovits 2005). Once *S. subterranea* is established in a soil, it is difficult to get rid of it, and it remains an important source of disease-producing inoculum even following long crop rotations. Both pathogens can survive many years in soils (Calvert 1968; Kritzman and Grinstein 1991). The multiplex real-time PCR developed in this study may serve as a sensitive and fast method to assess seed tubers and help growers avoid introduction of these pathogens to their farms and to detect these pathogens in soil.

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